Insect Molecular Biology

Insect Molecular Biology (2013) doi: 10.1111/imb.12076

Signalling properties and pharmacology of a 5-HT$_7$-type serotonin receptor from *Tribolium castaneum*

R. Vleugels, C. Lenaerts, J. Vanden Broeck and H. Verlinden

Laboratory for Molecular Developmental Physiology and Signal Transduction, Department of Animal Physiology and Neurobiology, Zoological Institute, KU Leuven, Leuven, Belgium

Abstract

In the last decade, genome sequence data and gene structure information on invertebrate receptors has been greatly expanded by large sequencing projects and cloning studies. This information is of great value for the identification of receptors; however, functional and pharmacological data are necessary for an accurate receptor classification and for practical applications. In insects, an important group of neurotransmitter and neurohormone receptors, for which ample sequence information is available but pharmacological information is missing, are the biogenic amine G protein-coupled receptors (GPCRs). In the present study, we investigated the sequence information, pharmacology and signalling properties of a 5-HT$_7$-type serotonin receptor from the red flour beetle, *Tribolium castaneum* (*Trica*5-HT$_7$). The receptor encoding cDNA shows considerable sequence similarity with cognate 5-HT$_7$ receptors and phylogenetic analysis also clusters the receptor within this 5-HT receptor group. Real-time reverse transcription PCR demonstrated high expression levels in the brain, indicating the possible importance of this receptor in neural processes. *Trica*5-HT$_7$ was dose-dependently activated by 5-HT, which induced elevated intracellular cyclic AMP levels but had no effect on calcium signalling. The synthetic agonists, α-methyl 5-HT, 5-methoxytryptamine, 5-carboxamidotryptamine and 8-hydroxy-2-(dipropylamino)tetralin hydrobromide, showed a response, although with a much lower potency and efficacy than 5-HT. Ketanserin and methiothepin were the most potent antagonists. Both showed characteristics of competitive inhibition on *Trica*5-HT$_7$. The signalling pathway and pharmacological profile offer important information that will facilitate functional and comparative studies of 5-HT receptors in insects and other invertebrates. The pharmacology of invertebrate 5-HT receptors differs considerably from that of vertebrates. The present study may therefore contribute to establishing a more reliable classification of invertebrate 5-HT receptors.

Keywords: biogenic amine, cyclic AMP, G protein-coupled receptor (GPCR), insect, pharmacology, red flour beetle.

Correspondence: Jozef Vanden Broeck, Zoological Institute, Naamsestraat 59 box 2465, 3000 Leuven, Belgium. Tel.: + 32 16 324260; fax: + 32 16 323902; e-mail: jozef.vandenbroeck@bio.kuleuven.be

Introduction

The red flour beetle, *Tribolium castaneum*, is known worldwide as a pest insect of stored products and, in recent years, it gained increasing interest as a model organism. It belongs to the order of Coleoptera, which is the largest and most species-diverse of all known metazoan orders. *T. castaneum* also serves as a research model for other (holometabolous) insects, contributing to the comparative study of genetic, developmental and physiological changes during evolution. The genome of *T. castaneum* was sequenced during 2004–2006 and its analysis was published in 2008 (Tribolium Genome Sequencing Consortium, 2008).

With more insect genomes becoming available, general features and differences between these genomes can be studied with increasing detail. One of the largest categories of proteins encoded in these genomes are the G protein-coupled receptors (GPCRs), which play a prominent role in many essential signalling pathways throughout the animal kingdom. A subgroup of these receptors are the biogenic amine GPCRs. For *Drosophila melanogaster* (Brody & Cravchik, 2000; Vanden Broeck, 2001; Hauser *et al*., 2006, 2008), *Apis mellifera* (Hauser *et al*., 2006),

Anopheles gambiae (Hill *et al*., 2002) and *Bombyx mori* (Fan *et al*., 2010), respectively 21, 19, 18 and 16 biogenic amine GPCRs were predicted. In *T. castaneum* 20 biogenic amine GPCRs are predicted (Hauser *et al*., 2008; Bai *et al*., 2011). In all metazoans, biogenic amines play a crucial role in many key processes. In insects, five major amines are involved in intercellular signalling: serotonin (5-hydroxytryptamine, 5-HT), dopamine, histamine, octopamine and tyramine. Serotonin is a monoamine neurotransmitter, known for its role in the regulation of many important human processes, such as mood and emotion, sleep, appetite, pain, sexual activity and learning abilities. In several insect species, similar processes were shown to be influenced by 5-HT, including sleep and circadian rhythms, nutrition, behaviour, development, learning and memory formation (Berridge & Patel, 1968; Trimmer, 1985; Cohen *et al*., 1988; Adachi *et al*., 1989; Chiang *et al*., 1992; Colas *et al*., 1995; Novak *et al*., 1995; Yuan *et al*., 2005, 2006; Walz *et al*., 2006; Dierick & Greenspan, 2007; Sitaraman *et al*., 2008, 2012; Anstey *et al*., 2009; Thamm *et al*., 2010; Ott *et al*., 2012).

Searches for receptor genes in publicly available genome databases are merely based on sequence information, which can provide indications for receptor classification and comparison; however, sequence data do not allow a very accurate prediction of the pharmacological and signalling properties or functional characteristics of these receptors. In insects, high sequence similarity has been observed between 5-HT receptors from different species, but still very little is known about their pharmacology and signalling cascade. This information is of great importance for functional studies. In addition, it would be very useful to examine the evolution of 5-HT receptors. All this information would contribute to proper invertebrate 5-HT receptor classification. Indeed, vertebrate 5-HT receptors have a clear classification based on sequence similarities, gene organization, second messenger coupling and pharmacological properties. They are divided into seven main classes. Six of these are GPCRs and the sole exception, 5-HT_3, is a ligand-gated ion channel. Several of these main receptor types probably result from ancient gene duplications in the common ancestor of vertebrates and invertebrates, therefore, this general classification has thus far been employed for invertebrate 5-HT receptors as well. Based on sequence similarities with vertebrate receptors, the insect receptors can all be classified as 5-HT_1-, 5-HT_2- and 5-HT_7-type GPCRs. Within the same receptor type, the signalling properties seem to be very well conserved between invertebrates and vertebrates, but their pharmacological characteristics may differ significantly (Tierney, 2001). In the genome of *T. castaneum*, four 5-HT receptors are predicted: two 5-HT_1 receptors and one 5-HT_2 and 5-HT_7 receptor (Hauser *et al*., 2008). Only one *Trica*5-HT_1 receptor has been characterized extensively (Vleugels *et al*., 2013). Orthologues of these four 5-HT receptors were characterized in *D. melanogaster* for some time. In addition, the existence of a second 5-HT_2 receptor had been suggested (Clark *et al*., 2004; Blenau & Thamm, 2011) and was recently confirmed experimentally (Gasque *et al*., 2013). In *A. mellifera*, two 5-HT_2 receptors are predicted as well, but only one 5-HT_1 and 5-HT_7 receptor were characterized (Witz *et al*., 1990; Saudou *et al*., 1992; Colas *et al*., 1995; Schlenstedt *et al*., 2006; Thamm *et al*., 2010).

The present study describes a pharmacological study of a 5-HT_7 receptor of *T. castaneum*, and the elucidation of its second messenger signalling. 5-HT_7-type serotonin receptors are characterized by positive coupling to cyclic AMP (cAMP) via binding to G_s proteins. Initial cloning of the human and rat 5-HT_7 receptor was published in 1993 by several laboratories independently (Bard *et al*., 1993; Lovenberg *et al*., 1993; Ruat *et al*., 1993; Shen *et al*., 1993). In insects, 5-HT_7 receptors were identified and pharmacologically characterized in *D. melanogaster* (Witz *et al*., 1990), *A. mellifera* (Schlenstedt *et al*., 2006), *Aedes aegypti* (Pietrantonio *et al*., 2001; Lee & Pietrantonio, 2003) and *Calliphora vicina* (Roser *et al*., 2012); however, no general consensus pharmacological profile could be composed from these studies, indicating the importance of more profound and detailed characterization studies of other insect (or other invertebrate) 5-HT receptors. The aim of the present study was to gain pharmacological and signalling information, in addition to the existing sequence information, concerning the 5-HT_7-type receptor of *T. castaneum*. These data will be important for generating a more detailed pharmacological classification system of insect 5-HT receptors and could be used in future functional assays and in practical applications.

Results and discussion

Receptor sequence and phylogenetic analysis of Trica*-5-HT$_7$*

The full-length sequence encoding a 5-HT_7-type receptor of *T. castaneum*, named *Trica*5-HT_7, was amplified from whole body cDNA. The open reading frame contains 1446 nucleotides, encoding a protein of 481 amino acids (Fig. 1) and a calculated molecular weight of 54.37 kDa. The amplified sequence corresponds to the predicted sequence on Beetlebase (National Center for Biotechnology Information (NCBI) reference XM_961484) (Hauser *et al*., 2008). Transmembrane (TM) topology prediction (using PHOBIUS; phobius.sbc.su.se/) revealed the presence of seven TM segments (TM1–7), the hallmark of all GPCRs. The large third intracellular loop, characteristic for biogenic amine receptors, contains four consensus sites for phosphorylation by protein kinase C (PKC) (S/T-x-R/

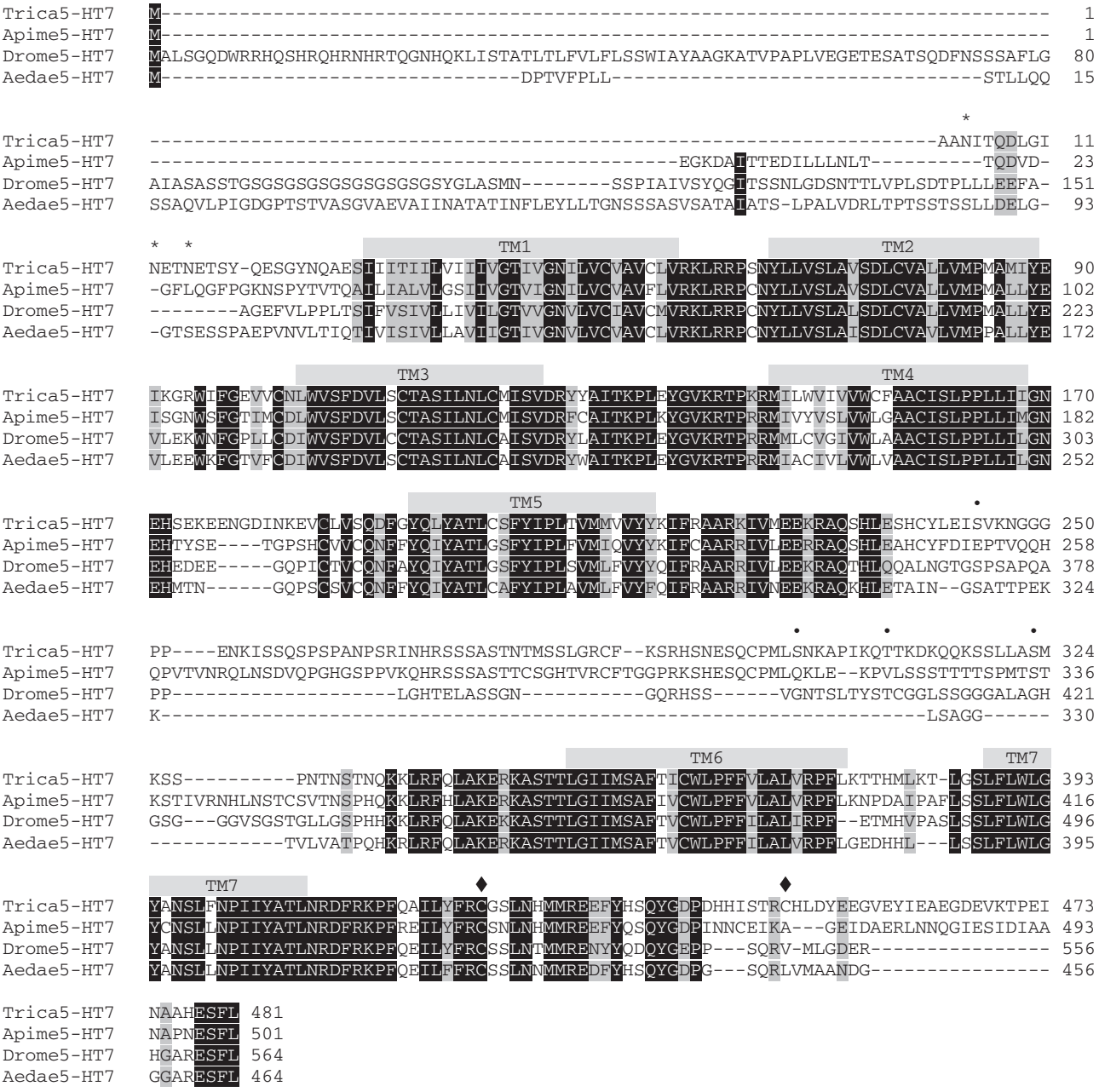

Figure 1. Amino acid sequence alignment of *Trica*5-HT$_7$ (NCBI Reference XP_966577). Aligment against sequences of orthologous receptors from *Apis mellifera* [*Apime*5-HT7; GenBank CAJ28210, (Schlenstedt *et al.*, 2006)], *Drosophila melanogaster* [*Drome*5-HT7; GenBank M55533, (Witz *et al.*, 1990)] and *Aedes aegypti* [*Aedae*5-HT7; GenBank AAG49292, (Pietrantonio *et al.*, 2001)]. Dashes indicate gaps that were introduced to maximize similarities. Identical residues between orthologous sequences are shown as white characters against black background, and conservative substitutions are shaded. Putative transmembrane domains are indicated by grey bars above the sequences (TM1-7). Asterisks (⋆) indicate putative N-linked glycosylation sites in the N-terminal region, dots (●) indicate putative phosphorylation sites for PKC, and diamonds (◆) indicate possible palmitoylation sites. Amino acid position numbers are indicated at the right. Alignment was performed using the multiple sequence alignment program MAFFT (version 7) (Katoh *et al.*, 2002, 2005).

K). The intracellular C-terminal region contains two cysteines for potential post-translational palmitoylation, while the extracellular N-terminus contains three consensus sites for glycosylation (N-x-[S/T]). Also other known 5-HT receptors possess several consensus sites for putative post-translational modifications. Not much is known about the actual post-translational modifications of most insect 5-HT receptors, although the 5-HT$_7$ receptor of *A. mellifera* was shown to be highly glycosylated (Schlenstedt *et al.*, 2006).

General Family-A GPCR characteristics are present in the amino acid sequence, such as the $D^{3.49}R^{3.50}Y^{3.51}$ sequence at the start of the second intracellular loop (numbering according to the Ballesteros-Weinstein system (Ballesteros & Weinstein, 1995)). This highly conserved tripeptide sequence is believed to have a key role in receptor activation (Bockaert & Pin, 1999; Gether, 2000). Several other properties of aminergic receptors are present, such as the consensus sequence $F^{6.44}$-x-x-x-$W^{6.48}$-x-$P^{6.50}$ in TM6 followed by a pair of phenylalanine residues, and the combination of a conserved aspartic acid in TM3 ($D^{3.32}$) and tryptophan in TM7 ($W^{7.40}$). The negatively charged side chain of $D^{3.32}$ is thought to interact with the protonated amine moiety of amine ligands. The conserved $N^{7.49}P^{7.50}$-x-x-$Y^{7.53}$ motif in TM7 is present as well. Mutagenesis experiments along this motif have been shown to affect the receptor's ligand affinity, G protein coupling, receptor signalling, desensitization and internalization (Barak *et al*., 1994; Gales *et al*., 2000; Gripentrog *et al*., 2000; Bouley *et al*., 2003; Fritze *et al*., 2003; Kalatskaya *et al*., 2004; Johnson *et al*., 2006; Borroto-Escuela *et al*., 2011). As other insect 5-HT_7 receptors, *Trica*5-HT_7 contains the sequence ESFL at its C-terminus. This corresponds to the consensus motif of class I PDZ-binding domains (E-[S/T]-x-Φ; with Φ being a hydrophobic amino acid, mostly V, L or I). PDZ domains are protein–protein interaction domains that play a role in protein targeting and protein complex assembly; however, no interactions with scaffolding proteins have as yet been reported for insect 5-HT receptors.

Comparison of the *Trica*5-HT_7 amino acid sequence with other 5-HT receptor orthologues revealed high sequence similarities to cognate insect 5-HT_7 receptors. The overall amino acid similarity (identical and conservatively substituted amino acids) compared with 5-HT_7 sequences of other insects was 74.4% for *A. mellifera* (Schlenstedt *et al*., 2006), 70.0% for *D. melanogaster* (Witz *et al*., 1990) and 66.3% for *Ae. aegypti* (Pietrantonio *et al*., 2001). The similarity within the TM regions is even higher (*A. mellifera*, 93.3% similarity; *D. melanogaster*, 94.5% similarity; and *Ae. aegypti*, 95.1% similarity). The overall amino acid sequence similarity to human 5-HT_7 splice variants ranges from 52.8% to 54.9%. Within the TM regions, similarity of *Trica*5-HT_7 was 68.6% with all human isoforms. These percentages are similar to the ones observed when other characterized insect amine receptors are compared with their orthologues in other species or taxa.

A multiple sequence alignment of different invertebrate and human 5-HT receptor sequences was used to construct a phylogenetic tree (Fig. 2). This phylogenetic analysis allows the evolutionary grouping of the three known invertebrate 5-HT receptor types. *Trica*5-HT_7 clusters with other known insect and human 5-HT_7 receptors. Highest similarity of the receptor was shown with the 5-HT_7 receptor of *A. mellifera*. Based on this available sequence information, insects seem to possess one 5-HT_7-type serotonin receptor, but may have two 5-HT_1 (*e.g. D. melanogaster* and *T. castaneum*) and/or two 5-HT_2 receptors (*e.g. D. melanogaster* and *A. mellifera*). This again shows that additional information on receptor pharmacology and downstream signalling may be of great importance to more accurately describe and classify invertebrate 5-HT receptors.

Receptor transcript distribution

Quantitative reverse transcription (RT)-PCR was used to compose a general tissue distribution profile. Expression levels were higher in the head than in the gut, the fat body and the reproductive system (Fig. 3A). More detailed study of the head indicated the highest expression in the brain (without optic lobes), and about half of this transcript level was observed in the optic lobes (Fig. 3B). This distribution is not surprising since serotonin is considered an important neurotransmitter. In *C. vicina*, 5-HT_7 receptor RT-PCR also showed highest expression in the brain (Roser *et al*., 2012). In *A. mellifera, in situ* hybridization showed expression of the *Apime*5-HT_7 receptor in all major regions of forager brains (Schlenstedt *et al*., 2006). Expression in different regions of the brain, such as the optic lobes, the antennal lobes and the mushroom bodies might indicate a role in neural pathways involved in processing sensory information and learning. Also other 5-HT receptors are shown to be expressed in insect brains (Schlenstedt *et al*., 2006; Thamm *et al*., 2010; Troppmann *et al*., 2010; Blenau & Thamm, 2011; Roser *et al*., 2012; Vleugels *et al*., 2013). Expression of *Trica*5-HT_7 in the salivary glands was relatively low compared with brain tissue, and was about the same as in the gut. Transcript levels in the gut might be due to expression in nervous tissue associated with the digestive tract. In *Ae. aegypti*, the 5-HT_7 receptor has been localized in axons that run in parallel with the hindgut. Since anti-5-HT antibodies showed no reactivity in the hindgut, 5-HT is probably not released locally, but rather acts as a hormone on the hindgut (Pietrantonio *et al*., 2001). In *Ae. aegypti*, *A. mellifera* and *C. vicina*, expression was also observed in the Malpighian tubules.

Functional information on the role of insect 5-HT receptors is still very limited. In *Ae. aegypti*, expression of 5-HT_7 in the tracheolar cells associated with the Malpighian tubules indicates a possible role for this receptor in respiration. *Apime*5-HT_1 has been suggested to mediate reduced positive phototactic behaviour in the honeybee (Thamm *et al*., 2010), while the negative effect on food intake of the 5-HT receptor antagonist methiothepin in *Drosophila* larvae was shown to be mediated by the

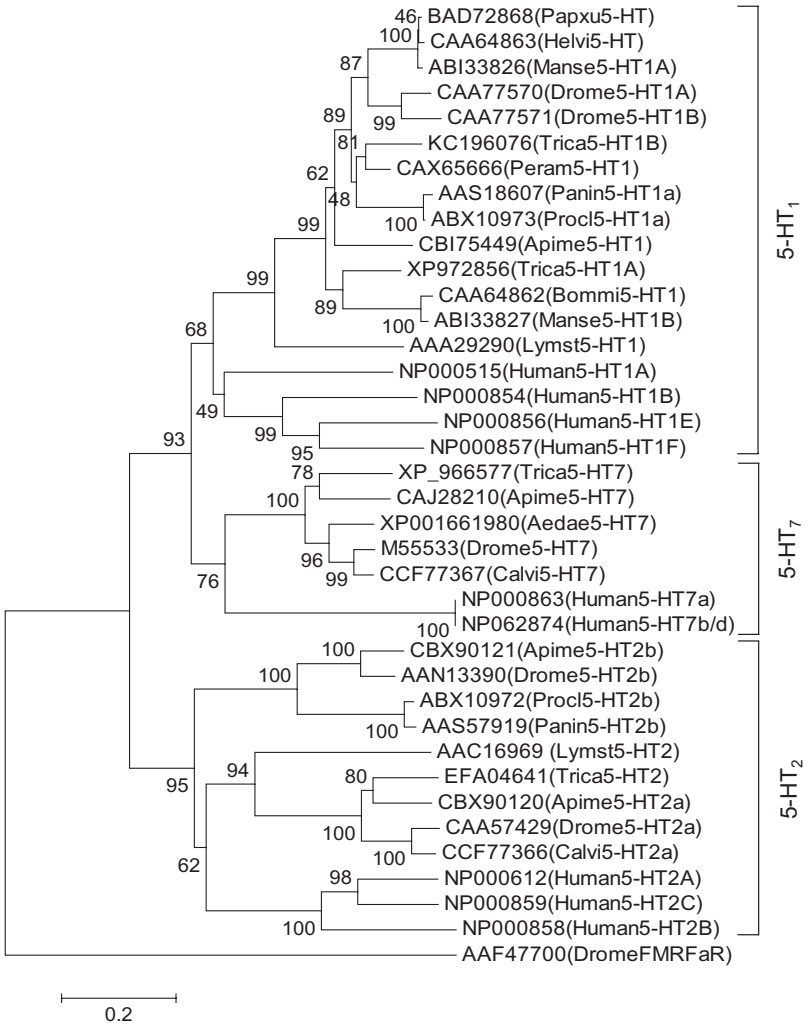

Figure 2. Maximum-likelihood tree of various invertebrate and human 5-HT receptors. Alignment was performed with amino acid sequences from TM1-5 and TM6-7. GenBank accession numbers are indicated in the tree, followed by the receptor name between brackets. The FMRFamide receptor of *Drosophila melanogaster* (isoform A) was used as outgroup. Phylogenetic and molecular evolutionary analyses were conducted by MEGA version 5.10 (Jones–Taylor–Thornton substitution model). Bootstrap values are based on 5000 replicates and are indicated on the nodes. The scale bar allows conversion of branch lengths in the dendogram to genetic distances between clades (0.2–20% genetic distance). Abbreviations used: Papxu, *Papilio xuthus*; Helvi, *Heliothis virescens*; Manse, *Manduca sexta*; Drome, *Drosophila melanogaster*; Trica, *Tribolium castaneum*; Peram, *Periplaneta americana*; Panin, *Panulirus interruptus*; Procl, *Procambarus clarkii*; Apime, *Apis mellifera*; Bommi, *Bombyx mori*; Lymst, *Lymnea stagnalis*; Aedae, *Aedes aegypti*; Calvi, *Calliphora vicina*.

5-HT$_{2A}$ receptor (Gasque *et al.*, 2013). More functional studies, however, must be performed to assign clear functions to these receptors.

Cell-based analysis of receptor pharmacology

Agonists. To examine receptor pharmacology, we employed a cell line stably expressing the promiscuous $G_{\alpha 16}$ protein to measure agonist-mediated receptor activation. Neither non-transfected cells, nor cells transfected with empty pcDNA3.1D vector responded to 5-HT (results not shown). After transfection with different quantities of *Trica*5-HT$_7$/pcDNA3.1D vector, no signal could be measured in non-agonist stimulated cells. The effects of 5-HT and the possible 5-HT receptor agonists αm-5-HT, 5-CT, 5-MT and 8-OH-DPAT were measured in cells transfected with *Trica*5-HT$_7$/pcDNA3.1D vector. High doses (100 μM) of the synthetic agonists activated the receptor, but only half as much as 5-HT (Fig. 4A). The dose–response relationship was examined for 5-HT with concentrations ranging from 1 pM to 1 mM. The resulting sigmoidal dose–response curve shows receptor activation in a dose-dependent and saturable manner (Fig. 4B). Maximum activation was achieved with 5-HT concentrations ≥5 μM. Half-maximum activation (EC_{50}) was obtained at 5-HT concentrations of 27.3 nM ($logEC_{50}$ =

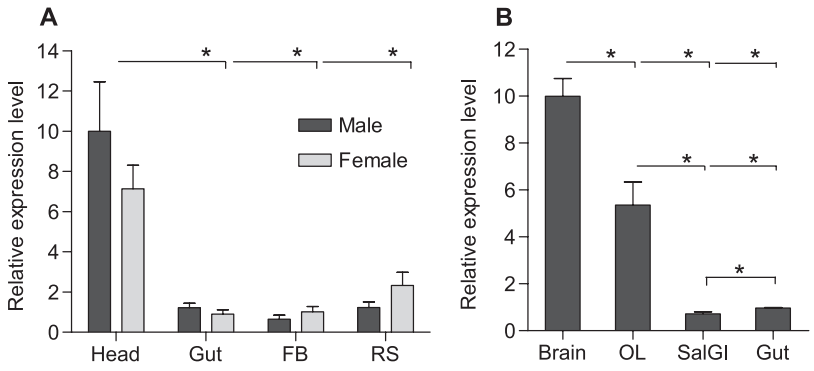

Figure 3. Expression profiles of transcripts encoding *Trica*5-HT$_7$ in sexually mature beetles. The data represent mean values of (A) three independent samples of 30 x heads, 50 x guts, 20 x fat body and 50 x reproductive system; and (B) three independent samples of 15 beetles each; run in duplicate ± SEM, normalized relative to RPS3 (ribosomal protein subunit 3), RPS18 and actin transcript levels. Statistically significant differences are indicated by asterisks above the bars ($P \leq 0.05$) (Kruskal–Wallis, IBM SPSS Statistics 20). Abbreviations: FB, fat body; RS, reproductive system; Brain, brain without the optic lobes; OL, optic lobes; SalGl, salivary glands.

–7.56 ± 0.04, mean ± SEM). For other insect 5-HT$_7$ receptors, EC$_{50}$ values were in the nanomolar range as well (1.06–1.75 nM in *A. mellifera*; 4 nM in *C. vicina*; 39.5 nM in *Ae. aegypti*) (Lee & Pietrantonio, 2003; Schlenstedt *et al.*, 2006; Roser *et al.*, 2012). Also the characterized 5-HT$_1$-type receptor of *T. castaneum* (*Trica*5-HT$_1$) had a comparable EC$_{50}$ value (Vleugels *et al.*, 2013). The biogenic amines dopamine, octopamine and tyramine did not induce detectable responses at concentrations up to 100 μM (results not shown), confirming that we characterized a specific serotonin (5-HT) receptor.

The dose–response relationship for potential agonists was measured the same way with concentrations from 100 pM to 1 mM. Since the efficacy achieved by any agonist depends on the number of receptors expressed, we measured a dose–response curve for 5-HT in every experiment and normalized all agonist effects to the maximum 5-HT response (Fig. 4B). The EC$_{50}$ values of the synthetic agonists in the micromolar range indicate that they are considerably less potent than 5-HT (Table 1). The mammalian 5-HT$_2$ receptor agonist αm-5-HT is most potent with an EC$_{50}$ value of 5.19 μM (logEC$_{50}$ = –5.29 ± 0.21), although still almost 200-fold less potent than 5-HT. Responses produced by the highest concentration of the synthetic agonists were also consistently weaker than the 5-HT-evoked responses (Fig. 4). High concentrations (1 mM) of 8-OH-DPAT, a partial and selective agonist for mammalian 5-HT$_1$ and 5-HT$_7$ receptors, resulted in 60% activation compared with a maximum level of stimulation obtained with 5-HT. The efficacies of the other agonists were below 50% of the maximum response of 5-HT. Especially the non-selective mammalian 5-HT receptor agonist, 5-MT, and the selective agonist for mammalian 5-HT$_1$ and 5-HT$_7$ receptors, 5-CT, showed

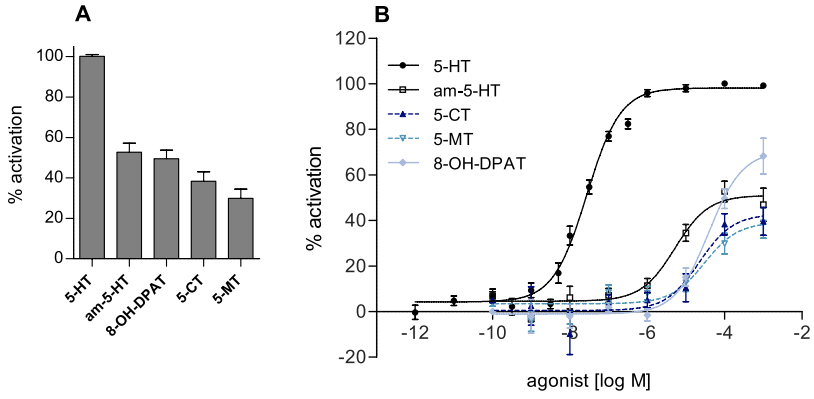

Figure 4. Effect of various possible 5-HT receptor agonists on *Trica*5-HT$_7$ in CHO-WTA11 cells. (A) Receptor activation in CHO-WTA11 cells after stimulation with 100 μM of agonist, shown as the percentage of activation achieved with 100 μM of 5-HT (indicated as 100%). (B) Dose–response curves with receptor activation shown as the percentage of activation achieved with 1 mM 5-HT (indicated as 100%). Data represent the mean ± SEM of 11 independent measurements in duplicate for 5-HT; six independent measurements (three in duplicate, three in triplicate) for 5-MT and 5-CT; seven independent measurements (four in duplicate, three in triplicate) for αm-5-HT and 8-OH-DPAT.

Table 1. EC_{50} values of agonists for *Trica*5-HT_7 receptor activation in CHO-WTA11 cells

Agonist	EC_{50}, μM	log(EC_{50}, mean ± SEM
5-HT	0.0273	−7.56 ± 0.042
αm-5-HT	5.19	−5.29 ± 0.21
5-CT	22.28	−4.65 ± 0.28
5-MT	30.28	−4.52 ± 0.28
8-OH-DPAT	38.75	−4.41 ± 0.15

only poor responses compared with 5-HT. Low efficacies and partial agonism were also observed in other insects. For example, in *A. mellifera*, 5-CT was shown to be a potent agonist of *Apime*5-HT_7 (EC_{50} = 24.3–40.0 nM), although its efficacy was about four times lower than 5-HT (Schlenstedt *et al.*, 2006). For *Trica*5-HT_1, the synthetic agonists also showed lower potencies and efficacies than 5-HT. On Trica5-HT1, the agonist αm-5-HT was both the most potent (with an EC_{50} value ~10.7 μM) and most efficient (~80% of the 5-HT response) (Vleugels *et al.*, 2013).

Mammalian 5-HT_7 receptors have the unique pharmacological profile with a rank order of potency 5-CT > 5-HT > 8-OH-DPAT (Gerhardt & van Heerikhuizen, 1997); however, none of the synthetic agonists seems to be as potent as 5-HT on *Trica*5-HT_7, nor on other insect 5-HT_7 receptors (Witz *et al.*, 1990; Lee & Pietrantonio, 2003; Schlenstedt *et al.*, 2006; Roser *et al.*, 2012). On *Apime*5-HT_7, the agonist 5-CT was only little less potent than 5-HT, while 8-OH-DPAT was only a poor agonist (Schlenstedt *et al.*, 2006). In *C. vicina*, 5-HT was a much stronger agonist of the *Calvi*5-HT_7 receptor than its analogue 5-CT, which in its turn was much more active than 8-OH-DPAT (Roser *et al.*, 2012). For most insect 5-HT_7 receptors, the rank order of potencies for these agonists thus appears to be: 5-HT > 5-CT > 8-OH-DPAT; however, the 5-HT_7 receptors of *Ae. aegypti* showed only weak activity upon stimulation with 5-CT or 8-OH-DPAT (Lee & Pietrantonio, 2003). It also has to be mentioned that the ergot derivate R(+)-lisuride was as potent as 5-HT in activating *Calvi*5-HT_7 (Roser *et al.*, 2012); however, its activity will probably not be restricted to 5-HT_7 receptors, since it is also known as an agonist of mammalian dopamine D2 receptors and several other types of 5-HT receptors.

Antagonists. Potential inhibition by synthetic antagonists was measured by simultaneously applying nanomolar concentrations of 5-HT (around the EC_{50} value) and a high dose of antagonist (100 μM). All tested antagonists decreased the 5-HT response (Fig. 5A) and dose-dependent relationships were investigated with antagonist concentrations ranging from 100 nM to 1 mM. Ketanserin, a selective antagonist of mammalian 5-HT_2 receptors, and methiothepin, a non-selective antagonist of mammalian 5-HT and dopamine receptors were most potent in inhibiting the agonist induced response on *Trica*5-HT_7 elicited at a 5-HT concentration of $10^{-7.5}$ M (= 31.6 nM; ~ EC_{50} value) (Fig. 5B). Their half-maximum

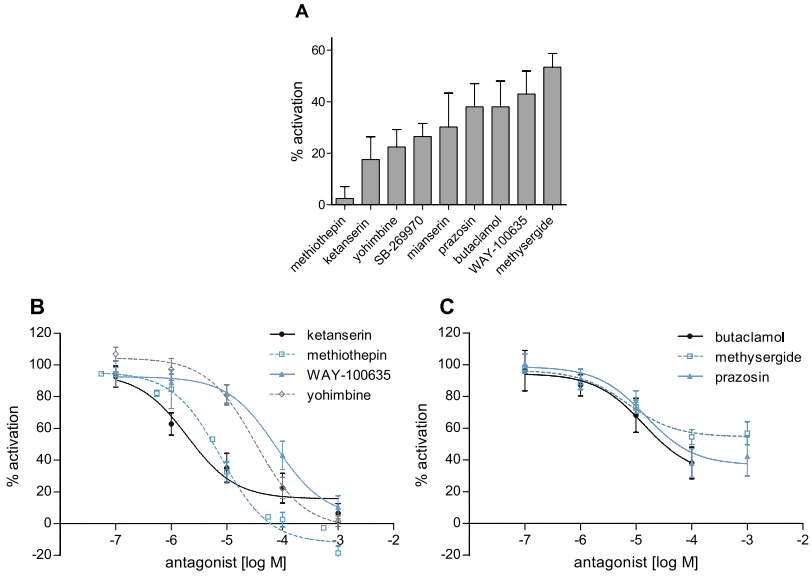

Figure 5. Effect of various possible 5-HT receptor antagonists on *Trica*5-HT_7 stimulated with 5-HT ($10^{-7.5}$ M) in CHO-WTA11 cells. (A) Effect of 100 μM antagonist shown as the percentage of activation achieved with $10^{-7.5}$ M (= 31.6 nM; ~ EC_{50} value) of 5-HT (indicated as 100%). (B and C) Dose–response curves with receptor activation shown as the percentage of activation achieved with $10^{-7.5}$ M 5-HT (indicated as 100%). Cells treated with BSA-medium only were used to define the basal level of 0%. Data represent the mean ± SEM of three (butaclamol and metysergide) or four (ketanserin) measurements in triplicate; or three measurements in triplicate and two (methiothepin and yohimbine) or three (prazosin, SB-269970 and WAY-100635) measurements in duplicate, or two (mianserin) measurements (one in duplicate, one in triplicate).

Table 2. IC_{50} values of antagonists for *Trica*5-HT_7 receptor activation in CHO-WTA11 cells (at $10^{-7.5}$ M 5-HT concentration)

Antagonist	IC_{50}, μM	log(IC_{50}, mean ± SEM
Ketanserin	1.95	–5.71 ± 0,25
Methysergide	6.98	–5.16 ± 0,27
Methiothepin	7.91	–5.10 ± 0,14
Butaclamol	13.65	–4.87 ± 0,44
Prazosin	14.82	–4.83 ± 0,30
SB-269970	15.82	–4.80 ± 0,21
Yohimbine	32.55	–4.49 ± 0,13
WAY-100635	76.80	–4.12 ± 0,18
Mianserin	Irrelevant to calculate	

inhibitory concentration (IC_{50}) values were 1.95 μM ($logIC_{50} = –5.71 \pm 0,25$) and 6.98 μM ($logIC_{50} = –5.16 \pm 0,27$), respectively. The IC_{50} values for all tested antagonists are indicated in Table 2. Ketanserin is known to be a moderate antagonist of *Calvi*5-HT_7, but was also shown to moderately inhibit activation of 5-HT_2 receptors in *D. melanogaster* (Colas *et al.*, 1995) and *C. vicina* (Roser *et al.*, 2012). Also in *Caenorhabditis elegans*, ketanserin was a rather potent inhibitor of the 5-HT_7 receptor SER-7 (Hobson *et al.*, 2003). Ketanserin did not show any detectable effects on *Trica*5-HT_1 (Vleugels *et al.*, 2013), but before using it as a selective blocker for *Trica*5-HT_7, it has to be confirmed that it does not act on other receptors in *T. castaneum*. Methiothepin on the other hand, was shown to be a potent inverse agonist of the constitutively active *Apime*5-HT_7 (Schlenstedt *et al.*, 2006), although the effect could not be calculated for *Calvi*5-HT_7. Membrane replacement studies also designate high pKi values for SER-7 of *C. elegans* (Hobson *et al.*, 2003). Methiothepin was also one of the most potent antagonists of *Trica*5-HT_1 (Vleugels *et al.*, 2013) and has been shown to moderately inhibit several other type 1 and type 2 5-HT receptors of insects and other invertebrates (Sugamori *et al.*, 1993; Colas *et al.*, 1995; Olde & McCombie, 1997; Angers *et al.*, 1998; Hamdan *et al.*, 1999; Barbas *et al.*, 2002; Troppmann *et al.*, 2010; Roser *et al.*, 2012). In *D. melanogaster*, methiothepin was even shown to antagonize all known 5-HT receptors (Gasque *et al.*, 2013), indicating that methiothepin indeed has a rather broad antagonistic activity.

The phenylpiperazine drug, WAY-100635, known to inhibit mammalian 5-HT_{1A} receptors, and the plant alkaloid, yohimbine, known to block mammalian α2-adrenergic receptors as well as locust tyramine receptors (Vanden Broeck *et al.*, 1995), exhibit clear dose-dependent inhibition on 5-HT induced *Trica*5-HT_7 (Fig. 5B). WAY-100635 has been shown to be an inverse agonist of the 5-HT_1 receptor of *Periplaneta americana* (Troppmann *et al.*, 2010). Yohimbine had only a moderate inhibitory effect on *Calvi*5-HT_7, but a higher antagonistic activity was measured for *Calvi*5-$HT_{2α}$. Both WAY-100635 and yohimbine thus do not seem very specific for a given insect receptor type. The dose–response curves of butaclamol (a mammalian dopamine receptor antagonist), methysergide (a 5-HT_2 receptor antagonist and partial 5-HT_1 agonist) and prazosin (a selective mammalian α1-adrenergic receptor antagonist) do not drop below 40% inhibition indicating rather low antagonistic efficiency (Fig. 5C). For butaclamol, a 1 μM concentration was not tested, since this could not be dissolved properly in the medium. Prazosin and methysergide were shown to be efficient and potent antagonists of *Trica*5-HT_1 (Vleugels *et al.*, 2013). Mianserin, a selective antagonist of mammalian 5-HT_2 receptors, shows no clear dose-dependent inhibition on *Trica*5-HT_7. Also for SB-269970 no pronounced dose-dependency could be observed, although known as a selective antagonist of mammalian 5-HT_7 receptors (Lovell *et al.*, 2000); however, more recently SB-269970 was also shown to block α2-adrenergic receptors (Foong & Bornstein, 2009). SB-269970 has been shown to be a very potent antagonist of *Calvi*5-HT_7, but only showed a weak inhibitory efficacy of about 15%. The highest inhibitory efficacy was 77% of the 5-HT response (in the presence of 6 nM 5-HT), and was achieved with clozapine. This is a monoaminergic antagonist with high affinity for several mammalian biogenic amine receptors including 5-HT_2 receptors; however, clozapine did not block *Apime*5-HT_7, on which SB-269970 also induced only poor antagonistic effects.

The two most potent antagonists that showed a dose-dependent response were investigated in more detail by looking at the dose–response relationship of 5-HT in the presence of different concentrations of antagonist (100 nM to 1 mM). Higher concentrations of antagonists resulted in a rightward shift of the 5-HT dose–response curves (Fig. 6) and consequently higher EC_{50} values for 5-HT activity. The efficacy of 5-HT did not change in presence of the antagonists. The Gaddum/Schild plot was used to examine the nature of inhibition and to calculate a pA2 value (i.e. a negative logarithm of the concentration of antagonist that doubles the amount of 5-HT required for obtaining the same effect). The slope of the Schild plot for methiothepin was 0.93 (0.84–1.01; 95% CI) and methiothepin can thus be considered a true competitive antagonist of *Trica*5-HT_7. The pA2 value of methiothepin was 6.50 (± 0.16). The Schild slope for ketanserin was much lower than 1, which indicates that the rightward shift of the dose–reponse curves was less than predicted by competitive inhibition; however, the main characteristics of competitive inhibition are present: a rightward shift of the dose–response curves of 5-HT with increasing concentration of antagonist, and the plateau for maximum 5-HT response was maintained, even at high antagonist concentrations. Taken together, ketanserin and methiothepin seem to be potent antagonists of *Trica*5-HT_7. Although

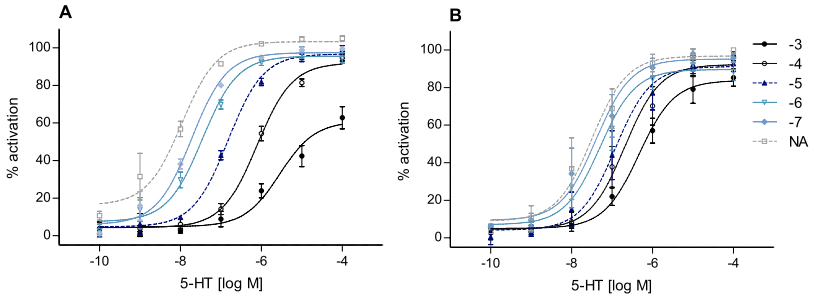

Figure 6. Dose-dependent activation of *Trica*5-HT$_7$ with 5-HT (0.1 nM–100 μM) in CHO-WTA11 cells, in the presence of different concentrations of antagonist (100 nM–1 mM). (A) methiothepin and (B) ketanserin. Receptor activity is shown as the percentage of activation achieved with 100 μM of 5-HT in the absence of antagonist (set at 100%). Cells treated with BSA-medium only were used to define the basal level of 0%. Data represent the mean ± SEM of two independent measurements (each performed in duplicate). NA, no antagonist.

methiothepin is not selective, since it also interacts with *Trica*5-HT$_1$ in the same concentration range as with *Trica*5-HT$_7$ (Vleugels *et al.*, 2013). The selectivity of ketanserin needs to be verified in studies with other 5-HT (and biogenic amine) receptors of *T. castaneum* (and other insects), especially 5-HT$_2$. In *C. vicina*, ketanserin indeed showed some inhibitory activity on *Calvi*5-HT$_2$.

The differences in pharmacological profiles between mammalian and insect receptors are probably attributable to the large evolutionary distance between protostomes and deuterostomes. Although the main receptor types were probably present in their common ancestor, a considerable divergence has occurred independently in receptors of mammals and insects. This may explain the existence of different receptor subtypes with specific pharmacological properties (Tierney, 2001). Selection during this evolution was probably based on functionally important receptor characteristics, such as ligand binding and G protein coupling, and this did not necessarily lead to conservation of recognition sites for man-made, synthetic ligands. Also, between different insects, differences in potency and efficacy can be observed for the agonists and antagonists that were originally designed for and applied to vertebrate (mainly mammalian and human) receptor types. Their specificity towards invertebrate (insect) receptors has never been profoundly tested or compared. It will therefore be of great interest to screen for synthetic compounds that more specifically target the insect receptor types.

Downstream signalling

CHO-PAM28 cells were used to determine whether *Trica*5-HT$_7$ couples to the Ca^{2+} signalling pathway. No effect of 5-HT was observed in these cells transfected with empty vector or *Trica*5-HT$_7$ containing vector. So we can conclude that the receptor does not couple via G$_q$ to the calcium-signalling pathway. In HEK293 cells, effects on the intracellular cAMP levels were examined by means of a CRE-based bioluminescent reporter assay. Basal levels of cAMP did not significantly change in cells transfected with an empty vector. Also no indications for a constitutive activity of the receptor were observed when comparing basal cAMP-dependent reporter levels (i.e. in the absence of 5-HT) in *Trica*5-HT7-expressing cells and in cells transfected with empty vector. There was also no inhibitory effect on NKH-477 (a forskolin analogue)-stimulated cyclic AMP levels; however, in cells expressing *Trica*5-HT$_7$, a dose-dependent increase in intracellular cAMP levels was measured upon stimulation with 5-HT (10 pM to 100 μM) (Fig. 7). Maximum response was obtained with 5-HT concentrations ≥10 μM. Half-maximum response levels were observed at 6.56 nM 5-HT (logEC_{50} = –8.18 ± 0.20, mean ± SEM). *Trica*5-HT$_7$ thus stimulates the cAMP production, probably via the G$_s$ protein. This is in

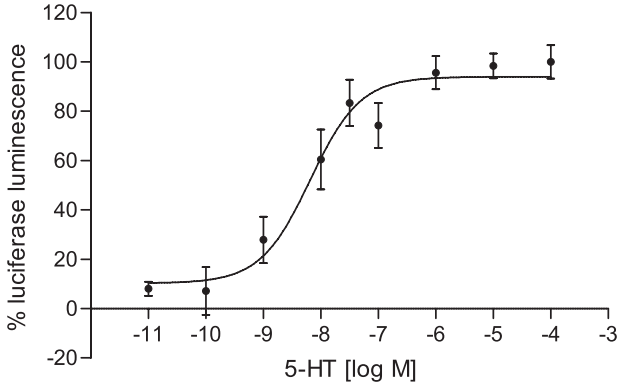

Figure 7. Dose-dependent effect of 5-HT (1 pM–100 μM) on the luciferase bioluminescence in HEK293 cells expressing *Trica*5-HT$_7$ as a result of changes in intracellular cAMP levels. Receptor activity is shown as the percentage of activation achieved with 100 μM of NKH-477 (set at 100%). Luciferase bioluminescence due to basal intracellular cAMP levels is set at 0%. The data represent the mean ± SEM of four independent measurements (one performed in duplicate, two in triplicate, and one in quadruplicate).

line with functional data for other 5-HT$_7$ receptors. The fact that both the amino acid sequence and the second messenger system are similar for 5-HT$_7$ receptors of both insects and vertebrates, is probably because 5-HT receptor types emerged from early gene duplications (followed by mutations and sequence drift) (Vernier *et al*., 1995). The small difference in EC$_{50}$ values for 5-HT measured in CHO and HEK cells, 27.3 and 6.6 nM respectively, might be explained by differences between both cell lines in the number of receptors exposed at the plasma membrane, in the levels of interacting proteins (including different G proteins), in the effector systems generating second messengers and/or in cellular metabolism. For example, the relative concentration of G proteins present in a given cell line might influence agonist-induced second messenger production (Bockaert *et al*., 1997). The different read out required for the different signals might also contribute to differences in EC$_{50}$ value. It should be noted here as well that possible imparities may exist between effects observed in cultured cell lines and physiological signalling processes occurring within the *in vivo* context of an organism (Bockaert *et al*., 1997; Kenakin, 1997).

Conclusions

Sequence information as well as second messenger coupling of the *Trica*5-HT$_7$ receptor resemble those of its vertebrate orthologues. The amino acid sequence of this receptor shows the characteristic features of biogenic amine GPCRs, as well as high similarity to other (insect) 5-HT$_7$ type serotonin receptors. As all known 5-HT$_7$ receptors, *Trica*5-HT$_7$ was dose-dependently activated by 5-HT, which induced elevated intracellular cAMP levels. These findings support other studies indicating that the main 5-HT receptor types result from early gene duplications in the common ancestor of insects and mammals.

By contrast, the known agonists and antagonists of mammalian 5-HT receptors do not seem to have the same selectivity on insect 5-HT receptors. The synthetic agonists, αm-5-HT, 5-CT, 5-MT and 8-OH-DPAT, were much less potent and efficient than 5-HT in activating *Trica*5-HT$_7$. From nine tested antagonists, ketanserin and methiothepin appeared to be most potent. In vertebrates, ketanserin is known as a selective antagonist of 5-HT$_2$ receptors, and methiothepin as a non-selective antagonist of both 5-HT and dopamine receptors.

The relatively low potencies of these synthetic ligands, as compared with 5-HT, and their differences in affinity for insect and mammalian receptors indicate the importance of profound pharmacological analyses of 5-HT receptor types in insects. Detailed study of insect 5-HT receptors is necessary to identify type-specific pharmacological ligands and to make an insect (or invertebrate) specific classification. For this, it may be necessary to screen for new compounds that efficiently target the insect receptor types. Specific stimulatory and inhibitory pharmacological agents will offer very helpful tools for functional studies and/or practical applications (e.g. research, medical use, pest management).

Experimental procedures

Drugs

The pharmacological ligands 3-hydroxytyramine (dopamine) hydrochloride, 5-carboxamidotryptamine maleate (5-CT), 5-HT hydrochloride (5-HT), 5-methoxytryptamine (5-MT), (±)-8-hydroxy-2-(dipropylamino)tetralin hydrobromide (8-OH-DPAT), α-methylserotonin maleate (αm-5-HT), (+)-butaclamol hydrochloride, ketanserin (+)-tartrate, methiothepin mesylate, methysergide maleate, mianserin hydrochloride, prazosin hydrochloride, DL-octopamine hydrochloride, SB-269970 hydrochloride (SB-269970 = (2R)-1-[(3-hydroxyphenyl)sulfonyl]-2 -(2-(4-methyl-1-piperidinyl)ethyl)pyrrolidine), tyramine hydrochloride, WAY-100635 maleate (WAY-100635 = N-(2-[4-(2-methoxyphenyl)-1-piperazinyl]-ethyl)-N-(2-pyridinyl) cyclohexanecarboxamide), and yohimbine hydrochloride were purchased from Sigma-Aldrich (St Louis, MO, USA).

Cloning of Trica*5-HT$_7$ receptor cDNA*

Oligonucleotide primers (sense primer 5′-CACCATGGCGGCC AATATAACTCAAGA-3′ and antisense primer 5′-TCATAGAAAC GATTCGTGTGCTGC-3′) were designed based on sequences available in Beetlebase released by the Human Genome Sequencing Center (Tcas_3.0; http://www.beetlebase.org/) (Hauser *et al*., 2008; Kim *et al*., 2010). The sequence, CACC, at the 5′ end of the sense primer is essential to enable directional cloning. The sequence encoding a *Trica*5-HT$_7$ receptor was amplified by means of PCR on whole body cDNA from adult *T. castaneum* beetles using Pwo DNA polymerase (Roche Applied Science, Indianapolis, IN, USA) and according to the protocol of the kit. The following PCR cycling programme was used: 94 °C for 2 min, followed by 30 cycles of [94 °C for 15 s, 65 °C for 30 s, 72 °C for 2 min], followed by a final elongation on 72 °C for 7 min. The PCR product was run on a 1% agarose gel, from which it was purified using the GenElute™ Gel Extraction Kit (Sigma-Aldrich). The purified DNA fragments were ligated into the directional pcDNA3.1D/V5-His-TOPO® expression vector (Invitrogen, Carlsbad, CA, USA). The vectors were then transformed in One Shot TOP10 chemically competent *Escherichia coli* cells (Invitrogen), which were grown overnight on 37 °C on lysogeny broth (LB) agar plates (35 g/l) with ampicillin (final concentration 50 μg/ml). Colonies were transferred to LB medium (25 g/l) with ampicillin (final concentration 50 μg/ml) and grown overnight on 37 °C in a horizontal shaker. Plasmids were isolated using the GenElute™ HP Plasmid Miniprep kit (Sigma Aldrich) and the inserted DNA sequences were verified on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) following the protocol outlined in the ABI PRISM BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems). Bacterial cells known to contain the correct receptor insert were grown at large scale in 100 ml LB broth medium (25 g/l) with ampicillin (final concentration 50 μg/ml). The expression vectors were subsequently isolated from these cells using

the EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany) according to the protocol recommended by the manufacturer.

Multiple sequence alignment and phylogenetic analysis

Amino acid sequences used for the sequence alignment were identified in the protein database of the NCBI with the amino acid sequence of *Trica*5-HT$_7$ as template. The multiple sequence alignment was performed with MAFFT (L-INS-i method) (Katoh *et al*., 2002, 2005). The alignment for the phylogenetic tree is based on the conserved regions TM1–5 and TM6–7, and was performed using MEGA software (version 5.10) (Tamura *et al*., 2011) using Muscle [MUltiple Sequence Comparison by Log-Expectation; (Edgar, 2004a, b)]. In MEGA, the genetic distance between sequences was calculated and a maximum likelihood tree was constructed using Jones–Taylor–Thornton amino acid substitution model. The reliability of the tree was estimated by 5000-fold bootstrap re-sampling.

Quantitative real-time PCR

For determination of expression levels of the receptor, tissues from sexually mature *T. castaneum* were dissected in phosphate buffered saline (PBS; NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.76 mM; pH 7.2), pooled and snap-frozen in liquid nitrogen. Tissues were subsequently homogenized and total RNA was extracted using the RNAqueous Micro Kit (Ambion, Austin, TX, USA) according to the protocol recommended by the kit, which included an additional DNase I digestion to degrade remaining contaminating genomic DNA. Total RNA was reverse transcribed into cDNA using SuperScriptIII reverse transcriptase (Invitrogen) as recommended by the manufacturer, and cDNA was diluted ten-fold before use. Transcript levels were quantified using the Fast Sybr Green assay kit (Applied Biosystems) in a StepOne Plus detection system (ABI Prism, Applied Biosystems). Primers were used in final concentrations of 500 nM: sense primer 5′-TGAAATCCTCGCCGAACAC-3′ and antisense primer 5′-TCGCTAGTTGAAAGCGAAGCT-3′ (Sigma-Aldrich). Other conditions were as recommended by the manufacturer. Reactions were run in duplicate and incubated on 50 °C for 2 min, followed by 95 °C for 10 min, followed by 40 cycles of [95 °C for 15 s and 60 °C for 1 min]. The specificity of the PCR products was assessed generating a dissociation curve (95 °C for 15 s, 60 °C for 1 min, and increase in temperature in 0.7 °C increments from 60 °C to 95 °C). Agarose gel electrophoresis of the PCR products confirmed the presence of a single band of the expected size and sequencing confirmed their identity. The relative quantity of target cDNA was quantified using the $\Delta\Delta C_T$-method including normalization to a calibrator on all PCR plates and an endogenous control. From a list of nine housekeeping genes (Supplementary table), ribosomal proteins RPS3 and RPS18 (with the ratio RP18/RP13 equal to 0.8), and actin were selected using geNorm (Vandesompele *et al*., 2002) as stable genes with respect to sex and tissue (Vuerinckx *et al*., 2011). These three genes were used as endogenous control to normalize all samples.

Cell culture and transfections

General binding studies were performed in Chinese hamster ovary (CHO-)WTA11 cells stably coexpressing apoaequorin and the promiscuous $G_{\alpha16}$ to determine the pharmacology independently of the downstream signalling. CHO-PAM28 cells stably expressing apoaequorin, but not the promiscuous $G_{\alpha16}$, and human embryonic kidney (HEK-)293 cells were employed to measure the functional signalling activity of the receptor via Ca^{2+} and cAMP, respectively.

All cells were cultured as monolayers in Dulbecco's Modified Eagle Medium Nutrient Mixture F12-Ham (DMEM/F12, Invitrogen) supplemented with 1% penicillin/streptomycin (10 000 units/ml penicillin and 10 mg/ml streptomycin in 0.9% NaCl) (Invitrogen) to prevent bacterial contamination of gram-positive and gram-negative bacteria, respectively. For CHO-WTA11 cells, 250 μg/ml zeocin (Invitrogen) was added and for CHO-PAM28 cells, 5 μg/ml puromycin dihydrochloride (Invitrogen) was added. The medium for CHO cells was supplemented with 10% fetal calf serum (inactivated at 65 °C, Sigma-Aldrich) and the medium for HEK293 cells was supplemented with 2% Ultroser G serum substitute (Pall Life Sciences). The cells were cultured in T25 flasks at 37 °C, in high relative humidity, with constant supply of 5% CO_2 and were subcultivated every 3 days at confluencies of 80–90%.

Transfections with either *Trica*5-HT$_7$/pcDNA3.1D or empty pcDNA3.1D vector were performed at *ca.* 60% confluency. For CHO cells, transfection reagent was prepared combining 2.5 ml Opti-MEM®I (Invitrogen), 5 μg plasmid DNA and 12.5 μl Plus™reagent (Invitrogen), stored at room temperature for 5 min and next repleted with 30 μl Lipofectamine™LTX (Invitrogen). After 30 min incubation at room temperature, the transfection mixture was added dropwise to the cells together with 5 ml medium. HEK293 cells were similarly cotransfected with 4 μg receptor construct and 2 μg CRE-luciferase construct (consisting of the open reading frame of the luciferase gene, downstream of a multimerized cAMP response element). Subsequently, the cells were grown overnight, next supplemented with 15 ml of medium for an additional overnight incubation period.

Bioluminescent assays

Transfected CHO cells were detached 2 days after transfection with PBS containing 0.2% EDTA and collected in DMEM/F-12 (without phenol red, with L-glutamine and 10 mM HEPES) (Gibco, Paisley, UK) to determine the amount of viable cells using a NucleoCounter® NC-100+™ (Chemometec, Alloroed, Denmark). Cells were next pelleted at 1240 *g* for 4 min at room temperature and resuspended in BSA-medium (DMEM/F12 without phenol red, with L-glutamine and 10 mM HEPES, supplemented with 1% bovine serum albumin) to a concentration of 5×10^6 cells/ml. Coelenterazine H (Invitrogen) was added to a final concentration of 5 μM, allowing the aequorin holoenzyme to be reconstituted. Cells were gently shaken for 4 h at room temperature in the dark. After a 10-fold dilution in BSA-medium, cells were incubated another 30 min. After incubation, receptor activity was measured as the light emission after injecting 50 μl of the cell suspension in wells with an equal volume of BSA-medium containing the dissolved pharmacological ligand (50 μl agonist solution, or 25 μl antagonist supplemented with 25 μl 5-HT solution). Since the efficacy depends on the number of receptors expressed, we measured a dose–response curve for 5-HT in every experiment and normalized all effects to this 5-HT response. Light emission was monitored during 30 s using a Mithras LB940 Microplate Reader (Berthold Technologies, Bad Wildbad, Germany). Cells were subsequently lysed with Triton

X-100 (2 μl per ml BSA-medium), serving as an internal reference. BSA medium was used as a negative control. Light emission from each well was calculated relative to the total response (ligand + Triton X-100) using the output file of Mikrowin2000 software (Mikrotek Laborsysteme, Overath, Germany). Further analysis was done in GRAPHPAD PRISM 5 (GRAPHPAD software).

Cotransfected HEK293 cells were detached and counted as described for the CHO cells, and next resuspended to a concentration of 10^6 cells/ml in DMEM/F12 medium containing 200 μM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich). In each well of a 96-well plate, 50 μl of the cell suspension was dispensed together with 50 μl of a pharmacological ligand dissolved in DMEM/F12 medium containing 200 μM IBMX. The water-soluble analogue of forskolin, NKH-477, was added to 20 μM concentrations when inhibitory effects on intracellular cAMP were measured. After incubation for 3–4 h in a CO_2 incubator at 37 °C, 100 μl of SteadyLite Plus (Perkin-Elmer, Boston, MA, USA) was added to each well and the plate was gently shaken for 15 min in the dark. Light emission resulting from the luciferase activity was measured for 5 s per well using a Mithras LB940 Microplate Reader (Berthold Technologies). Medium containing IBMX was used as a negative control. Data were analysed as described for CHO cells.

Acknowledgements

The authors thank Prof. Arnd Baumann (Research Centre Jülich, Germany) for providing the HEK293 cell line, and Marc Parmentier (University of Brussels, Belgium) and Michel Detheux (Euroscreen S.A., Belgium) for providing both CHO cell lines. They gratefully acknowledge the Interuniversity Attraction Poles (IAP) programme (Belgian Science Policy Grant P7/40) and the Flemish Research Foundation (FWO) and KU Leuven Research Foundation (GOA/11/02) for financial support. They also thank Joost Van Duppen for technical support and Dr Kristel Vuerinckx for her help with dissections and cDNA sample preparations. R.V and C.L. were supported by a PhD fellowship from IWT (Agency for Innovation by Science and Technology in Flanders). H.V. obtained a postdoctoral research fellowship from FWO (Research Foundation of Flanders).

References

Adachi, T., Takiya, S., Suzuki, Y., Iwami, M., Kawakami, A., Takahashi, S.Y. *et al*. (1989) cDNA structure and expression of bombyxin, an insulin-like brain secretory peptide of the silkmoth *Bombyx mori*. *J Biol Chem* **264**: 7681–7685.

Angers, A., Storozhuk, M.V., Duchaine, T., Castellucci, V.F. and DesGroseillers, L. (1998) Cloning and functional expression of an *Aplysia* 5-HT receptor negatively coupled to adenylate cyclase. *J Neurosci* **18**: 5586–5593.

Anstey, M.L., Rogers, S.M., Ott, S.R., Burrows, M. and Simpson, S.J. (2009) Serotonin mediates behavioral gregarization underlying swarm formation in desert locusts. *Science* **323**: 627–630.

Bai, H., Zhu, F., Shah, K. and Palli, S.R. (2011) Large-scale RNAi screen of G protein-coupled receptors involved in larval growth, molting and metamorphosis in the red flour beetle. *BMC Genomics* **12**: 388–398.

Ballesteros, J.A. and Weinstein, H. (1995) Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. *Methods Neurosci* **25**: 366–428.

Barak, L.S., Tiberi, M., Freedman, N.J., Kwatra, M.M., Lefkowitz, R.J. and Caron, M.G. (1994) A highly conserved tyrosine residue in G protein-coupled receptors is required for agonist-mediated beta 2-adrenergic receptor sequestration. *J Biol Chem* **269**: 2790–2795.

Barbas, D., Zappulla, J.P., Angers, S., Bouvier, M., Castellucci, V.F. and DesGroseillers, L. (2002) Functional characterization of a novel serotonin receptor (5-HTap2) expressed in the CNS of *Aplysia californica*. *J Neurochem* **80**: 335–345.

Bard, J.A., Zgombick, J., Adham, N., Vaysse, P., Branchek, T.A. and Weinshank, R.L. (1993) Cloning of a novel human serotonin receptor (5-HT7) positively linked to adenylate cyclase. *J Biol Chem* **268**: 23422–23426.

Berridge, M.J. and Patel, N.G. (1968) Insect salivary glands: stimulation of fluid secretion by 5-hydroxytryptamine and adenosine-3′,5′-monophosphate. *Science* **162**: 462–463.

Blenau, W. and Thamm, M. (2011) Distribution of serotonin (5-HT) and its receptors in the insect brain with focus on the mushroom bodies. Lessons from *Drosophila melanogaster* and *Apis mellifera*. *Arthropod Struct Dev* **40**: 381–394.

Bockaert, J. and Pin, J.P. (1999) Molecular tinkering of G protein-coupled receptors: an evolutionary success. *EMBO J* **18**: 1723–1729.

Bockaert, J., Brand, C. and Journot, L. (1997) Do recombinant receptor assays provide affinity and potency estimates? *Ann N Y Acad Sci* **812**: 55–70.

Borroto-Escuela, D.O., Romero-Fernandez, W., Garcia-Negredo, G., Correia, P.A., Garriga, P., Fuxe, K. *et al*. (2011) Dissecting the conserved NPxxY motif of the M3 muscarinic acetylcholine receptor: critical role of Asp-7.49 for receptor signaling and multiprotein complex formation. *Cell Physiol Biochem* **28**: 1009–1022.

Bouley, R., Sun, T.X., Chenard, M., McLaughlin, M., McKee, M., Lin, H.Y. *et al*. (2003) Functional role of the NPxxY motif in internalization of the type 2 vasopressin receptor in LLC-PK1 cells. *Am J Physiol Cell Physiol* **285**: C750–C762.

Brody, T. and Cravchik, A. (2000) *Drosophila melanogaster* G protein-coupled receptors. *J Cell Biol* **150**: F83–F88.

Chiang, R.G., Chiang, J.A. and Davey, K.G. (1992) A sensory input inhibiting heart-rate in an insect, *Rhodnius prolixus*. *Experientia* **48**: 1122–1125.

Clark, M.C., Dever, T.E., Dever, J.J., Xu, P., Rehder, V., Sosa, M.A. *et al*. (2004) Arthropod 5-HT2 receptors: a neurohormonal receptor in decapod crustaceans that displays agonist independent activity resulting from an evolutionary alteration to the DRY motif. *J Neurosci* **24**: 3421–3435.

Cohen, R.W., Friedman, G.P. and Waldbauer, G.P. (1988) Physiological control of nutrient self selection in *Heliothis zea* larvae: the role of serotonin. *J Insect Physiol* **34**: 935–940.

Colas, J.F., Launay, J.M., Kellermann, O., Rosay, P. and Maroteaux, L. (1995) *Drosophila* 5-HT2 serotonin receptor: coexpression with fushi-tarazu during segmentation. *Proc Natl Acad Sci U S A* **92**: 5441–5445.

Dierick, H.A. and Greenspan, R.J. (2007) Serotonin and neuropeptide F have opposite modulatory effects on fly aggression. *Nat Genet* **39**: 678–682.

Edgar, R.C. (2004a) MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* **5**: 113.

Edgar, R.C. (2004b) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* **32**: 1792–1797.

Fan, Y., Sun, P., Wang, Y., He, X., Deng, X., Chen, X. *et al.* (2010) The G protein-coupled receptors in the silkworm, *Bombyx mori*. *Insect Biochem Mol Biol* **40**: 581–591.

Foong, J.P. and Bornstein, J.C. (2009) 5-HT antagonists NAN-190 and SB 269970 block alpha2-adrenoceptors in the guinea pig. *Neuroreport* **20**: 325–330.

Fritze, O., Filipek, S., Kuksa, V., Palczewski, K., Hofmann, K.P. and Ernst, O.P. (2003) Role of the conserved NPxxY(x)5,6F motif in the rhodopsin ground state and during activation. *Proc Natl Acad Sci U S A* **100**: 2290–2295.

Gales, C., Kowalski-Chauvel, A., Dufour, M.N., Seva, C., Moroder, L., Pradayrol, L. *et al.* (2000) Mutation of Asn-391 within the conserved NPXXY motif of the cholecystokinin B receptor abolishes Gq protein activation without affecting its association with the receptor. *J Biol Chem* **275**: 17321–17327.

Gasque, G., Conway, S., Huang, J., Rao, Y. and Vosshall, L.B. (2013) Small molecule drug screening in *Drosophila* identifies the 5HT2A receptor as a feeding modulation target. *Sci Rep* **3**: srep02120.

Gerhardt, C.C. and van Heerikhuizen, H. (1997) Functional characteristics of heterologously expressed 5-HT receptors. *Eur J Pharmacol* **334**: 1–23.

Gether, U. (2000) Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocrinol Rev* **21**: 90–113.

Gripentrog, J.M., Jesaitis, A.J. and Miettinen, H.M. (2000) A single amino acid substitution (N297A) in the conserved NPXXY sequence of the human N-formyl peptide receptor results in inhibition of desensitization and endocytosis, and a dose-dependent shift in p42/44 mitogen-activated protein kinase activation and chemotaxis. *Biochem J* **352** (Pt 2): 399–407.

Hamdan, F.F., Ungrin, M.D., Abramovitz, M. and Ribeiro, P. (1999) Characterization of a novel serotonin receptor from *Caenorhabditis elegans*: cloning and expression of two splice variants. *J Neurochem* **72**: 1372–1383.

Hauser, F., Cazzamali, G., Williamson, M., Blenau, W. and Grimmelikhuijzen, C.J. (2006) A review of neurohormone GPCRs present in the fruitfly *Drosophila melanogaster* and the honey bee *Apis mellifera*. *Progress Neurobiol* **80**: 1–19.

Hauser, F., Cazzamali, G., Williamson, M., Park, Y., Li, B., Tanaka, Y. *et al.* (2008) A genome-wide inventory of neurohormone GPCRs in the red flour beetle *Tribolium castaneum*. *Front Neuroendocrinol* **29**: 142–165.

Hill, C.A., Fox, A.N., Pitts, R.J., Kent, L.B., Tan, P.L., Chrystal, M.A. *et al.* (2002) G protein-coupled receptors in *Anopheles gambiae*. *Science* **298**: 176–178.

Hobson, R.J., Geng, J., Gray, A.D. and Komuniecki, R.W. (2003) SER-7b, a constitutively active Galphas coupled 5-HT7-like receptor expressed in the *Caenorhabditis elegans* M4 pharyngeal motorneuron. *J Neurochem* **87**: 22–29.

Johnson, M.S., Robertson, D.N., Holland, P.J., Lutz, E.M. and Mitchell, R. (2006) Role of the conserved NPxxY motif of the 5-HT2A receptor in determining selective interaction with isoforms of ADP-ribosylation factor (ARF). *Cell Signal* **18**: 1793–1800.

Kalatskaya, I., Schussler, S., Blaukat, A., Muller-Esterl, W., Jochum, M., Proud, D. *et al.* (2004) Mutation of tyrosine in the conserved NPXXY sequence leads to constitutive phosphorylation and internalization, but not signaling, of the human B2 bradykinin receptor. *J Biol Chem* **279**: 31268–31276.

Katoh, K., Misawa, K., Kuma, K. and Miyata, T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res* **30**: 3059–3066.

Katoh, K., Kuma, K., Toh, H. and Miyata, T. (2005) MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Res* **33**: 511–518.

Kenakin, T. (1997) Differences between natural and recombinant G protein-coupled receptor systems with varying receptor/G protein stoichiometry. *Trends Pharmacol Sci* **18**: 456–464.

Kim, H.S., Murphy, T., Xia, J., Caragea, D., Park, Y., Beeman, R.W. *et al.* (2010) BeetleBase in 2010: revisions to provide comprehensive genomic information for *Tribolium castaneum*. *Nucleic Acids Res* **38**: D437–D442.

Lee, D.W. and Pietrantonio, P.V. (2003) In vitro expression and pharmacology of the 5-HT7-like receptor present in the mosquito *Aedes aegypti* tracheolar cells and hindgut-associated nerves. *Insect Mol Biol* **12**: 561–569.

Lovell, P.J., Bromidge, S.M., Dabbs, S., Duckworth, D.M., Forbes, I.T., Jennings, A.J. *et al.* (2000) A novel, potent, and selective 5-HT(7) antagonist: (R)-3-(2-(2-(4-methylpiperidin-1-yl)ethyl) pyrrolidine-1-sulfonyl) phen ol (SB-269970). *J Med Chem* **43**: 342–345.

Lovenberg, T.W., Baron, B.M., de Lecea, L., Miller, J.D., Prosser, R.A., Rea, M.A. *et al.* (1993) A novel adenylyl cyclase-activating serotonin receptor (5-HT7) implicated in the regulation of mammalian circadian rhythms. *Neuron* **11**: 449–458.

Novak, M.G., Ribeiro, J.M. and Hildebrand, J.G. (1995) 5-hydroxytryptamine in the salivary glands of adult female *Aedes aegypti* and its role in regulation of salivation. *J Exp Biol* **198**: 167–174.

Olde, B. and McCombie, W.R. (1997) Molecular cloning and functional expression of a serotonin receptor from *Caenorhabditis elegans*. *J Mol Neurosci* **8**: 53–62.

Ott, S.R., Verlinden, H., Rogers, S.M., Brighton, C.H., Quah, P.S., Vleugels, R.K. *et al.* (2012) Critical role for protein kinase A in the acquisition of gregarious behavior in the desert locust. *Proc Natl Acad Sci U S A* **109**: E381–E387.

Pietrantonio, P.V., Jagge, C. and McDowell, C. (2001) Cloning and expression analysis of a 5HT7-like serotonin receptor cDNA from mosquito *Aedes aegypti* female excretory and respiratory systems. *Insect Mol Biol* **10**: 357–369.

Roser, C., Jordan, N., Balfanz, S., Baumann, A., Walz, B., Baumann, O. *et al.* (2012) Molecular and pharmacological characterization of serotonin 5-HT2alpha and 5-HT7 receptors in the salivary glands of the blowfly *Calliphora vicina*. *PLoS ONE* **7**: e49459.

Ruat, M., Traiffort, E., Leurs, R., Tardivel-Lacombe, J., Diaz, J., Arrang, J.M. *et al.* (1993) Molecular cloning, characterization,

and localization of a high-affinity serotonin receptor (5-HT7) activating cAMP formation. *Proc Natl Acad Sci U S A* **90**: 8547–8551.

Saudou, F., Boschert, U., Amlaiky, N., Plassat, J.L. and Hen, R. (1992) A family of *Drosophila* serotonin receptors with distinct intracellular signalling properties and expression patterns. *EMBO J* **11**: 7–17.

Schlenstedt, J., Balfanz, S., Baumann, A. and Blenau, W. (2006) Am5-HT7: molecular and pharmacological characterization of the first serotonin receptor of the honeybee (*Apis mellifera*). *J Neurochem* **98**: 1985–1998.

Shen, Y., Monsma, F.J., Jr, Metcalf, M.A., Jose, P.A., Hamblin, M.W. and Sibley, D.R. (1993) Molecular cloning and expression of a 5-hydroxytryptamine7 serotonin receptor subtype. *J Biol Chem* **268**: 18200–18204.

Sitaraman, D., Zars, M., LaFerriere, H., Chen, Y.C., Sable-Smith, A., Kitamoto, T. *et al.* (2008) Serotonin is necessary for place memory in *Drosophila*. *Proc Natl Acad Sci U S A* **105**: 5579–5584.

Sitaraman, D., LaFerriere, H., Birman, S. and Zars, T. (2012) Serotonin is critical for rewarded olfactory short-term memory in *Drosophila*. *J Neurogenet* **26**: 238–244.

Sugamori, K.S., Sunahara, R.K., Guan, H.C., Bulloch, A.G., Tensen, C.P., Seeman, P. *et al.* (1993) Serotonin receptor cDNA cloned from *Lymnaea stagnalis*. *Proc Natl Acad Sci U S A* **90**: 11–15.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**: 2731–2739.

Thamm, M., Balfanz, S., Scheiner, R., Baumann, A. and Blenau, W. (2010) Characterization of the 5-HT1A receptor of the honeybee (*Apis mellifera*) and involvement of serotonin in phototactic behavior. *Cell Mol Life Sci* **67**: 2467–2479.

Tierney, A.J. (2001) Structure and function of invertebrate 5-HT receptors: a review. *Comp Biochem Physiol A Mol Integr Physiol* **128**: 791–804.

Tribolium Genome Sequencing Consortium (2008) The genome of the model beetle and pest *Tribolium castaneum*. *Nature* **452**: 949–955.

Trimmer, B.A. (1985) Serotonin and the control of salivation in the blowfly *Calliphora*. *J Exp Biol* **114**: 307–328.

Troppmann, B., Balfanz, S., Baumann, A. and Blenau, W. (2010) Inverse agonist and neutral antagonist actions of synthetic compounds at an insect 5-HT1 receptor. *Br J Pharmacol* **159**: 1450–1462.

Vanden Broeck, J. (2001) Neuropeptides and their precursors in the fruitfly, *Drosophila melanogaster*. *Peptides* **22**: 241–254.

Vanden Broeck, J., Vulsteke, V., Huybrechts, R. and De Loof, A. (1995) Characterization of a cloned locust tyramine receptor cDNA by functional expression in permanently transformed Drosophila S2 cells. *J Neurochem* **64**: 2387–2395.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. *et al.* (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**: RESEARCH0034.

Vernier, P., Cardinaud, B., Valdenaire, O., Philippe, H. and Vincent, J.D. (1995) An evolutionary view of drug-receptor interaction: the bioamine receptor family. *Trends Pharmacol Sci* **16**: 375–381.

Vleugels, R., Lenaerts, C., Baumann, A., Vanden Broeck, J. and Verlinden, H. (2013) Pharmacological characterization of a 5-HT1-type serotonin receptor in the red flour beetle, *Tribolium castaneum*. *PLoS ONE* **8**: e65052.

Vuerinckx, K., Verlinden, H., Lindemans, M., Vanden Broeck, J. and Huybrechts, R. (2011) Characterization of an allatotropin-like peptide receptor in the red flour beetle, *Tribolium castaneum*. *Insect Biochem Mol Biol* **41**: 815–822.

Walz, B., Baumann, O., Krach, C., Baumann, A. and Blenau, W. (2006) The aminergic control of cockroach salivary glands. *Arch Insect Biochem Physiol* **62**: 141–152.

Witz, P., Amlaiky, N., Plassat, J.L., Maroteaux, L., Borrelli, E. and Hen, R. (1990) Cloning and characterization of a *Drosophila* serotonin receptor that activates adenylate cyclase. *Proc Natl Acad Sci U S A* **87**: 8940–8944.

Yuan, Q., Lin, F., Zheng, X. and Sehgal, A. (2005) Serotonin modulates circadian entrainment in *Drosophila*. *Neuron* **47**: 115–127.

Yuan, Q., Joiner, W.J. and Sehgal, A. (2006) A sleep-promoting role for the *Drosophila* serotonin receptor 1A. *Curr Biol* **16**: 1051–1062.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Nucleotide sequences of primers for *Tribolium castaneum* housekeeping genes. Abbreviations: Act, actin; EF1α, elongation factor 1α; RPS, ribosomal protein subunit; Tub, tubulin; Ubq, ubiquitin.